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SIMPLE MECHANISMS FOR BROADSPECTRUM COLOR CONTROL IN AQUATIC ORGANISMS

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14. ABSTRACT Phenotypes of a freshwater zooplankton (*Diaptomus*) sampled from alpine and subalpine lakes exert control of color from red to blue. This broad-spectrum control is exerted over a single pigment material using two mechanisms; (1) esterification for fine control producing "shades" and (2) protein binding for broad-band control. Broad spectrum color change using control of a single pigment offers a fantastic opportunity to study photonic control in a simple but dynamic system. The goal of this project was to examine the mechanisms behind bathychromic color shift (red to blue) observed in freshwater alpine copepods. Specifically, we examined (1) environmental conditions which result in changes in pigmentation, (2) genes and proteins involved in red-blue color change, and (3) the role of pigment esters in color intensity. We sampled alpine lakes in the Rocky Mountains and determined that reproductive cycle is the biological driver for color change. We developed and adapted methods for pigment extraction and identification (LC-MS) in "low mass" tissue samples, fractionated more than a dozen ester compounds, and examined the distribution of pigment using microscopy and microRaman analysis. We have also isolated and manipulated blue carotenoprotein ex-vivo. Genomic and proteomic approaches were used to isolate proteins and mRNAs for sequencing.

15. SUBJECT TERMS
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Roberts Final Report – 2012

1. Principal Investigator Name:
Aaron Roberts

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SIMPLE MECHANISMS FOR BROADSPECTRUM COLOR CONTROL IN AQUATIC ORGANISMS

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Dr. Hugh Delong

7. Executive Summary: Phenotypes of a freshwater zooplankton (*Diaptomus*) sampled from alpine and subalpine lakes exert control of color from red to blue. This broad-spectrum control is exerted over a single pigment material using two mechanisms; (1) esterification for fine control producing "shades" and (2) protein binding for broad-band control. Broad spectrum color change using control of a single pigment offers a fantastic opportunity to study photonic control in a simple but dynamic system. The goal of this project was to examine the mechanisms behind bathychromic color shift (red to blue) observed in freshwater alpine copepods. Specifically, we examined (1) environmental conditions which result in changes in pigmentation, (2) genes and proteins involved in red-blue color change, and (3) the role of pigment esters in color intensity. We sampled alpine lakes in the Rocky Mountains and determined that reproductive cycle is the biological driver for color change. We developed and adapted methods for pigment extraction and identification (LC-MS) in "low mass" tissue samples, fractionated more than a dozen ester compounds, and examined the distribution of pigment using microscopy and microRaman analysis. We have also isolated and manipulated blue carotenoprotein ex-vivo. Genomic and proteomic approaches were used to isolate proteins and mRNAs for sequencing. Details of all experimental approaches are available in theses from Kovach and Hudelson (full citations below).

8. Objectives
The **goal** of this project was to understand the biological processes involved in color control in alpine *Diaptomus* and the use of a single carotenoid compound to achieve coloration (Figure 1). Specifically, the **objectives and approaches** were to:

(1) Determine the biological mechanisms behind fine control (shading) of red pigmentation in alpine copepods.

Approach: Examine the environmental factors (UVR; predation; temperature; etc.) that induce changes in red pigments in alpine copepods.

Approach: Investigate the roles of astaxanthin accumulation, esterification, and cellular distribution in determining pigment intensity.

(2) Determine the biological mechanisms behind broad-spectrum control of pigmentation in alpine copepods.

Approach: Examine environmental conditions which stimulate alpine copepods to change from predominantly red pigmentation to blue pigmentation.

Approach: Isolate and identify caroteno-protein complexes which alter the astaxanthin spectra from red reflected light to blue reflected light and determine their cellular distribution.

Figure 1. Alpine *Diaptomus shoshone* expressing different color patterns. Coloration shift from red to blue is achieved by protein binding to the carotenoid pigment astaxanthin.

9. Findings

We completed nearly all of the stated objectives and are continuing the final objective (protein sequencing). Findings are detailed below.

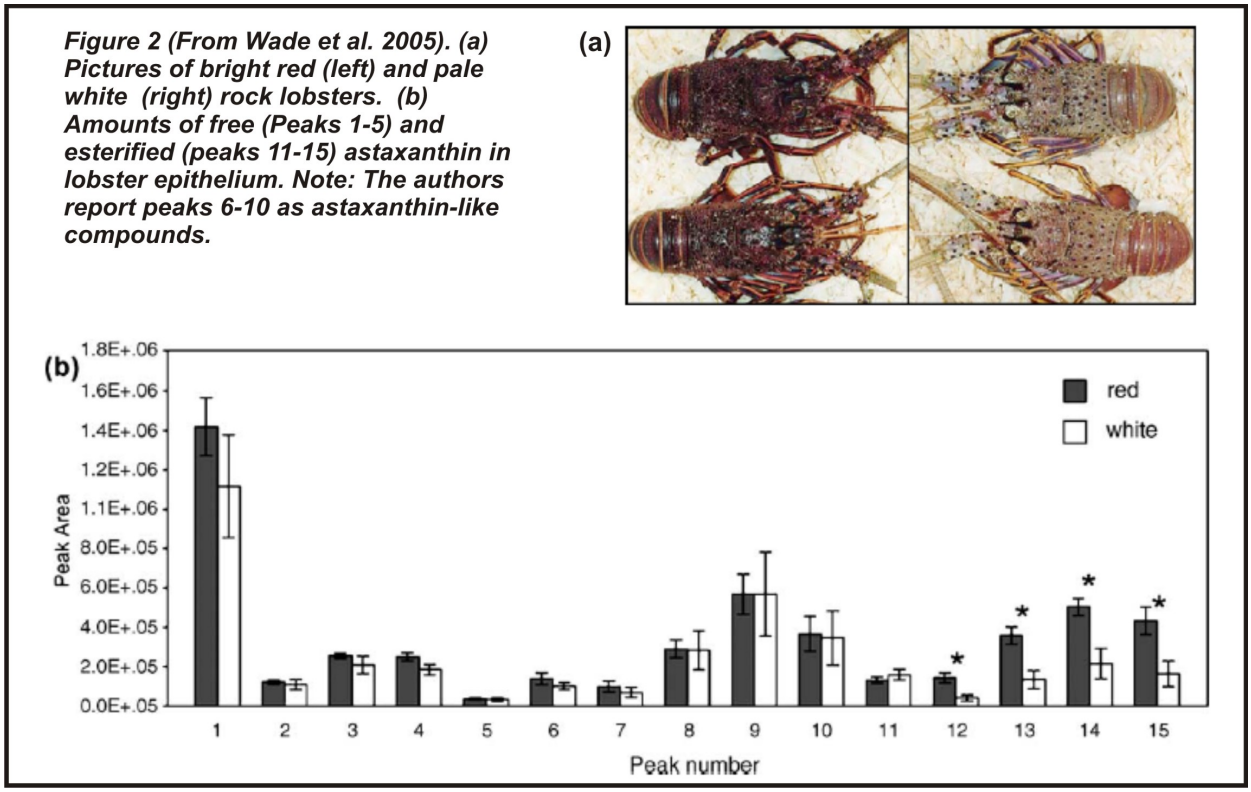
Objective 1: Biological mechanisms behind fine control of red pigmentation
Approach 1: Environmental factors inducing changes in red pigment intensity.

Progress: This objective is completed. The purpose of this approach was to insure that we would be able to manipulate red intensity in the organisms for laboratory experimentation in support of Approach 2 (mechanism examination). In year 1, we were able to reliably and repeatedly obtain copepods with a variety of red intensities from ponds in which ultraviolet radiation differed and predation pressure differed (by presence/absence of fish). We were also able to manipulate the intensity of that pigmentation using UV shading plastics in mesocosm (enclosed cages) experiments. Results of mechanistic analyses of these samples are discussed under Approach 2 and details can be found in Matthew Kovach's thesis (available through UNT libraries, reference below).

Approach 2: Investigate the roles of astaxanthin accumulation, esterification, and cellular distribution in determining pigment intensity.

Progress: This objective was completed. We have met the major objectives as presented in the proposal but began a new collaboration with Dr. Guido Verbeck (UNT Chemistry) using micro-Raman and nano-manipulator approaches to identifying organic compounds at the cellular level. The proposal outlined three approaches to examining pigment intensity: (1) examination of spectral properties of astaxanthin esters, (2) examination of cellular distribution of astaxanthin, and (3) identification of proteins/genes involved in pigment intensity changes. Briefly, previous reports in a marine crustacean by Wade et al. (2005) discuss findings that red intensity in lobster pigmentation is not based on total pigment accumulation, but rather the spectral properties of different pigment esters. Thus, two organisms with the same amount of total pigment but different ester composition have different red intensities (Figure 2). We hypothesized that changes in red intensity due to environmental stimuli might be due to biochemical conversion of ester compounds rather than accumulation of total pigment. We initially developed methods for determination of pigments in low biomass samples (see 2010 Annual Report). At the conclusion of that report, we stated that ongoing analyses included examination of the molar absorption of different esterified pigments. We found that regardless of the fatty acid conjugate, each pigment molecule carried the same molar absorbance resulting in similar shading intensity (Figure 3). These studies were carried out both using extracts from copepods as well as synthetic pigments separated using LC. This implies that the pigments themselves are not different but rather their distribution is being changed to result in differential coloration. This finding is supported by the differences between free, mono-, and di-esterified pigment content between different color morphs (Figure 4). Each of the forms has differences in lipophilicity thus altering its cellular distribution from cytosol to lipid droplets. This work was conducted by James Smith (Ph.D. student supported by the grant) who is preparing a manuscript describing this work to be submitted to *Comp Biochem Physiol*.

Figure 2 (From Wade et al. 2005). (a) Pictures of bright red (left) and pale white (right) rock lobsters. (b) Amounts of free (Peaks 1-5) and esterified (peaks 11-15) astaxanthin in lobster epithelium. Note: The authors report peaks 6-10 as astaxanthin-like compounds.



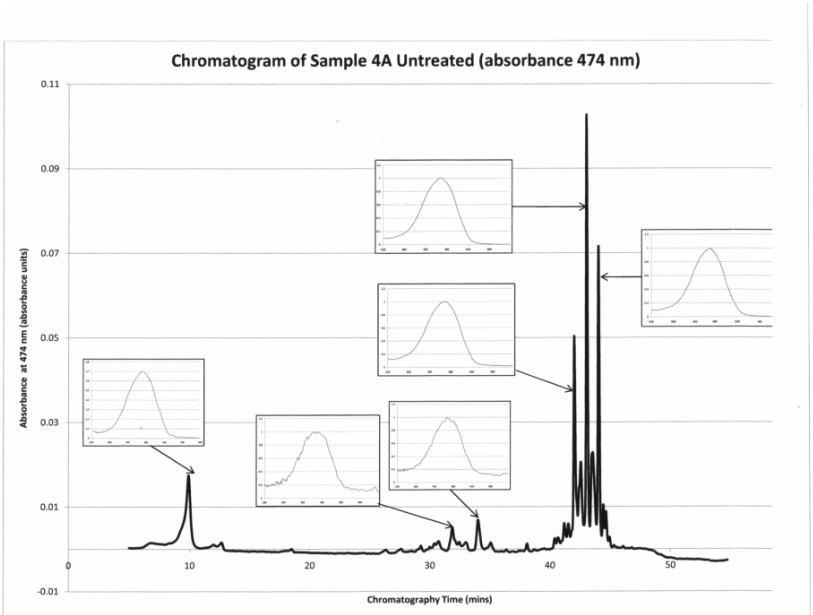


Figure 3. Sample chromatogram from copepod extracts. Each peak corresponds to a different pigment ester and cluster based on free (10 min), mono- (30 min), and di- (40 min) esterified. Smaller boxes are absorbance curves associated with each individual pigment. There are slight shifts in peak absorbance but we do not believe these contribute significantly to coloration differences.

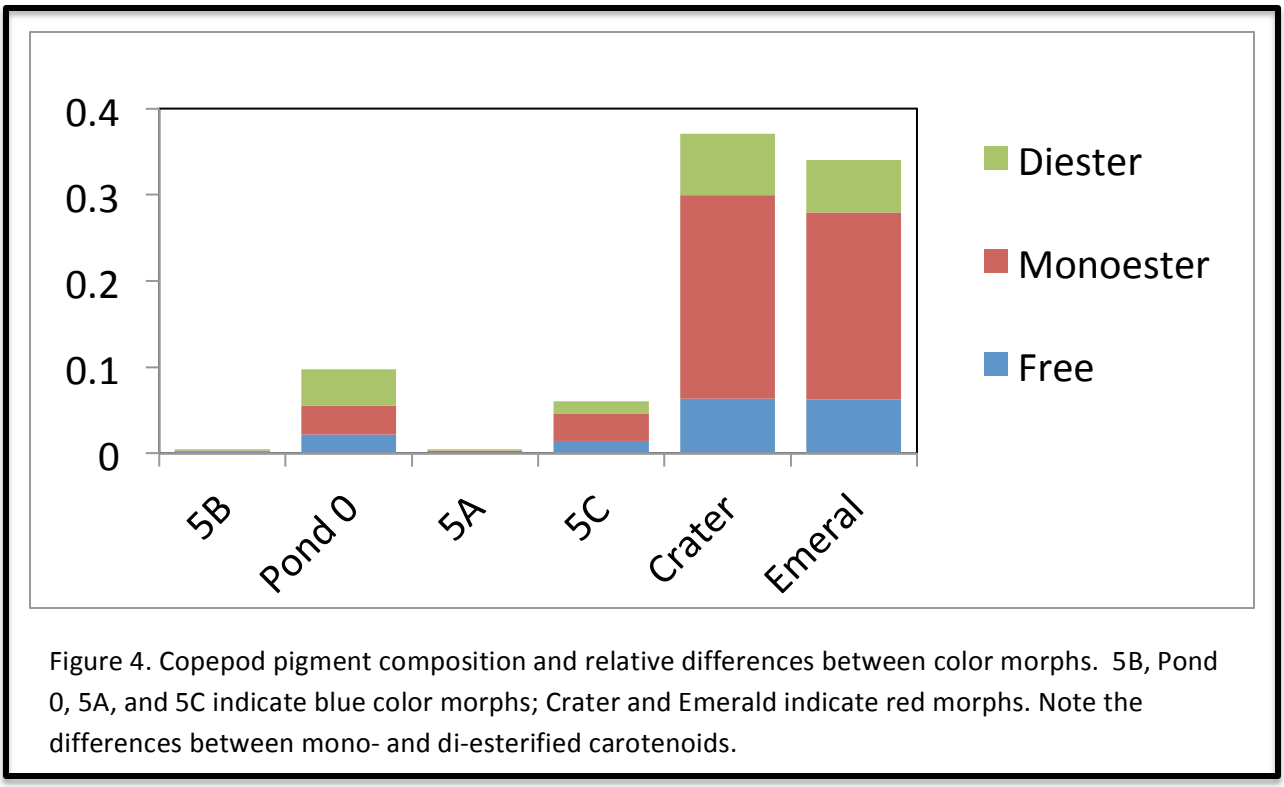
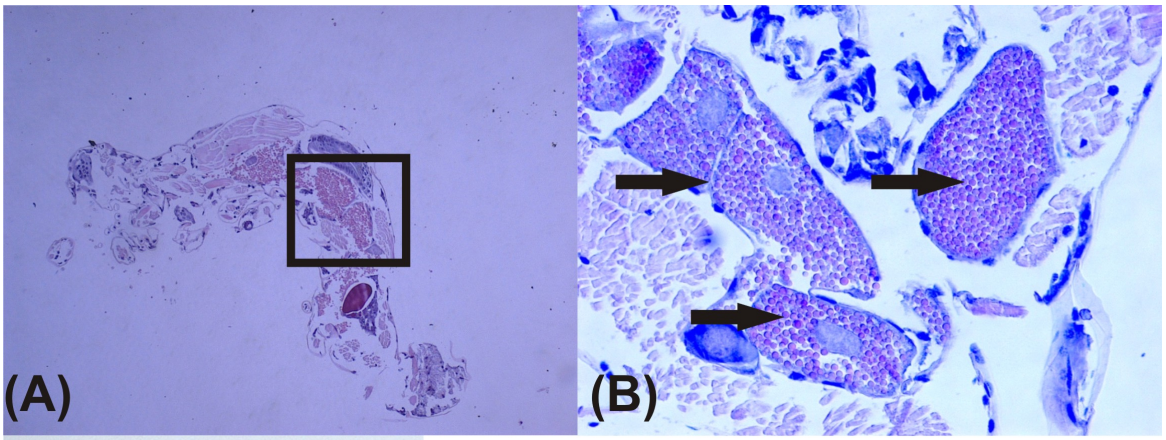


Figure 4. Copepod pigment composition and relative differences between color morphs. 5B, Pond 0, 5A, and 5C indicate blue color morphs; Crater and Emerald indicate red morphs. Note the differences between mono- and di-esterified carotenoids.

Toward examining the cellular distribution of astaxanthin, we prepared histological sections of copepods and found that most of the esterified astaxanthin (red) is located internally, particularly in the developing eggs of the ovaries regardless of the color pattern of the female (Figure 5). In fact, blue females have red ovaries and red egg cases. We hypothesized that this is to provide photoprotection and antioxidants (another property of carotenoid pigments) to the offspring until they are able to begin synthesizing their own pigments. This hypothesis is supported by work completed by Matthew Kovach and Karista Hudelson (master's students supported by this grant; Kovach and Hudelson theses available from UNT libraries). Kovach and Hudelson found that carotenoid content and color patterning affected copepod sensitivity to ultraviolet light as well as, for the first time, pro-oxidant chemicals. This work is detailed in two graduate theses and two manuscripts (in preparation).

Year 3 work: Much of the work thus far identifying different ester pigments in specific compartments has relied on histological techniques and crude extracts. We collaborated with Dr. Guido Verbeck (UNT Chemistry; AFOSR supported) using micro-Raman spectroscopy and nanomanipulator-coupled mass spectrometry to identify and quantify pigments in subcellular compartments (Figure 6 and 7). That work has already resulted in two meeting presentations and a manuscript in preparation (Fox et al.).



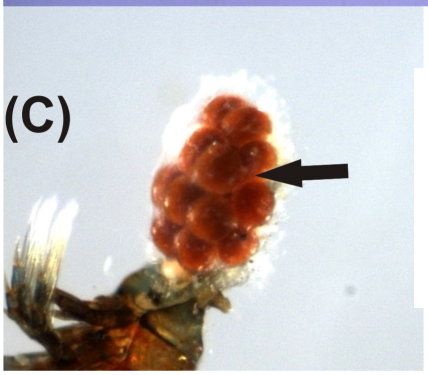
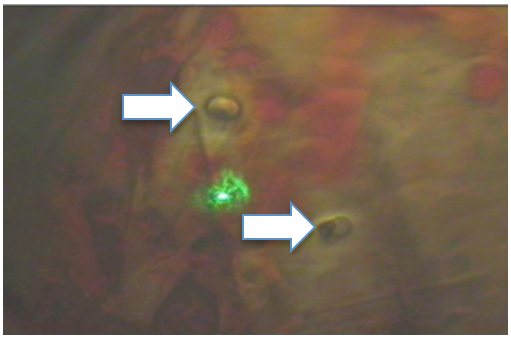


Figure 5. Histological preparations of copepods. (A) Longitudinal section indicating ovaries. (B) Ovarian tissue. Arrows indicate lipid globules in developing eggs containing astaxanthin. (C) Macroscopic picture of egg case; note deep red color of eggs (arrows).



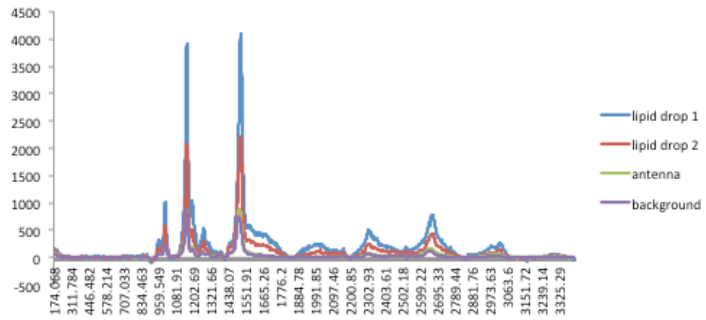


Figure 6. Micro-raman spectroscopy. (Top) Micrograph showing lipid droplets in copepod tissue (arrows). (Bottom) Raman spectrograph of pigments from individual compartments. Intensity of raman peaks indicate greater accumulation of pigment in specific cellular structures.

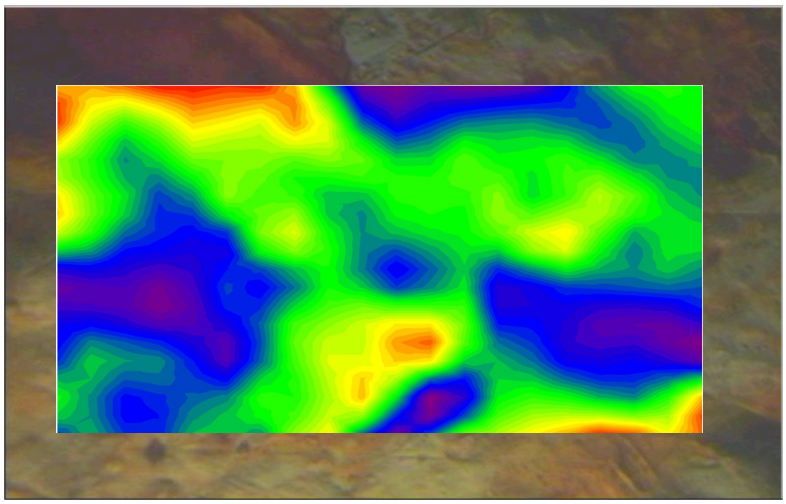


Fig 7. Micro-raman imaging of protein-bound and unbound pigment complexes in situ.

The final component of this approach is the identification of genes/proteins involved in ester conversion. Initial work on this objective began in year 2.

Objective 2. Determine the biological mechanisms behind broad-spectrum control of pigmentation in alpine copepods.

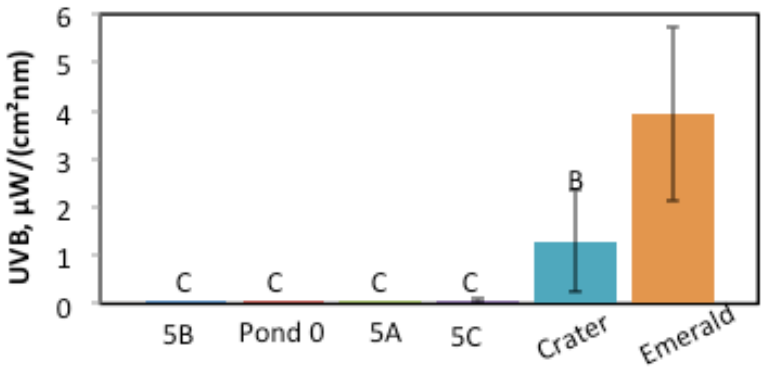
Approach 1: Examine environmental conditions which stimulate alpine copepods to change from predominantly red pigmentation to blue pigmentation.

Progress: This subobjective was completed. Blue pigmentation in marine crustaceans occurs through binding of various proteins to pigment molecules. We hypothesized that blue coloration in alpine copepods was achieved through a similar mechanism by binding of the protein crustacyanin to astaxanthin. Binding of the protein (crustacyanin) to the red pigment molecule (astaxanthin) results in a conformational shift of the aromatic rings on the pigment molecule and a change in light absorption/reflection.

Determination of environmental conditions which induce this shift was deemed critical in order to carry out laboratory manipulations of blue carotenoprotein complexes. Completion of this objective was an unexpected challenge. Organisms at the field sites regularly change between red and blue coloration. However, we were unable to induce this color change in the laboratory using temperature, salinity, pH, hardness, or UV manipulation. Interestingly, blue color shifts occur most dramatically in very shallow ponds. In deep lakes, we (and others) have observed behavioral avoidance of UV light as vertical migration in the water column. Organisms that are able to vertically migrate are always red. We have observed that in our systems where vertical migration is not possible, organisms undergo red to blue color change. Despite our lack of success in manipulating color using UV thus far, we continued to hypothesize that UV was involved as a cue, a focus of Year 2 fieldwork. However, results of those studies has shown that UV attenuation in the shallow ponds is surprisingly high and thus these organisms may not be

under a great deal of radiation-induced selective pressure (Figure 7). Work is continuing with Dr. Dmitri Deheyn (AFOSR funded) to examine UV-reflectance in these pigments.

Figure 7. UVB in selected copepod habitats. 5A-C and Pond 0 are blue copepod habitats; Crater and Emerald are red habitats. Blue habitats receive significantly less UVB than do red habitats.



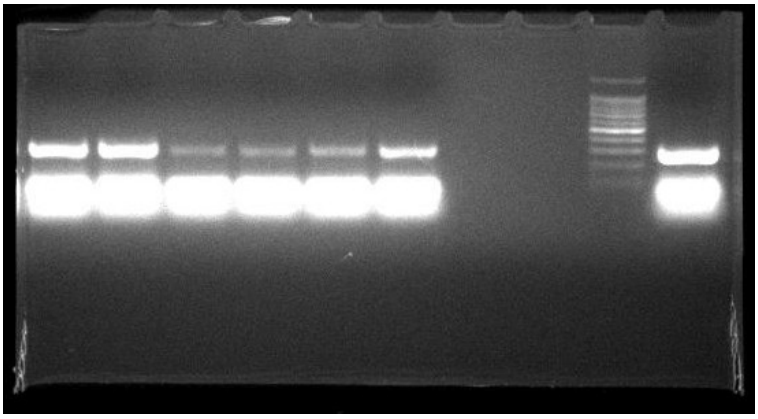
Alternatively, following discussions at the annual review meeting, we examined reproductive cycle as a cue. We found that, as discussed in the cellular distribution objectives, that esterified pigments were trafficked in lipid droplets and accumulated in the ovaries of gravid female copepods. As these pigments accumulated in the eggs, the females own color went from red to pale (almost translucent) to blue. We have been able to use maturation and reproductive status as a predictive tool for color in copepods.

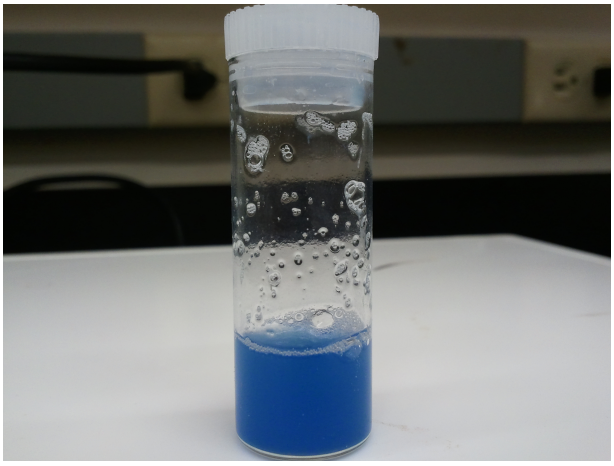
Approach 2: Isolate and identify caroteno-protein complexes which alter the astaxanthin spectra from red reflected light to blue reflected light and determine their cellular distribution.

Progress: During year 1 of the study, we made some crude progress in isolating the blue carotenoprotein intact and reversibly manipulating color (ex vivo) of the extract. We also successfully isolated mRNA and total protein from alpine copepods toward identifying proteins/genes involved in promoting color change. In year 2, we worked heavily on these objectives with varying success. We successfully obtained reproducible PCR products from primers designed to target crustacyanin genes. For some of these, particularly the crustacyanin alpha subunit, we also obtained differential expression between color morphs (Figure 8). We worked to improve primer binding and efficiencies to produce better and more complete gene sequence data. However, sequencing values between copepod crustacyanin and known marine crustaceans (largely lobster) did not improve indicating evolutionary divergence between freshwater and marine crustaceans. In the protein arena, we have used 2D gel – LCMS proteomic approaches to examine differences in protein expression between color morphs. This approach resulted in a large number of differentially expressed peptides and we released those samples for sequencing in a collaboration with 21st Century Biochemicals (company). To specifically target crustacyanin, we used a direct affinity column approach. Using lobster shell,

we developed methods to isolate and purify the crustacyanin complex and then re-solubilized it in buffer (Figure 9). We also adapated these methods for use in low-biomass copepod samples.

Figure 8. (Top) Bands showing PCR product corresponding to crustacyanin-alpha subunit. Note the differences in relative intensity. (Bottom) Crustacyanin carotenoprotein complex isolated and resolubilized. Much improved from initial crude extracts.





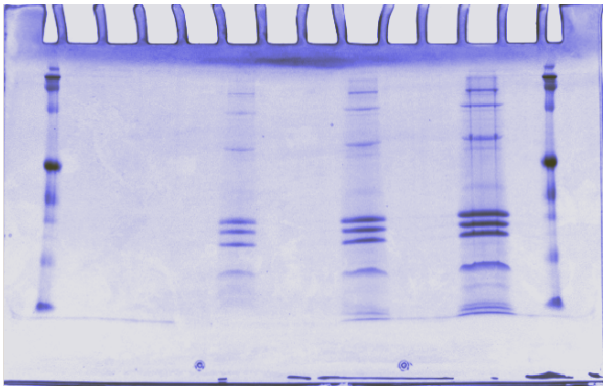


Figure 9. Isolated and resolubilized crustacyanin carotenoprotein comlex from lobster shell. Sample extraction and purification is MUCH improved from early extracts.

10. Supported Personnel

Support was provided to one faculty member and partial/complete support to five graduate students. In the initial proposal, funding was designated for a postdoctoral fellow. The postdoc candidate found a "permanent" position last minute and I designated part of that salary to a graduate student.

Dr. Aaron Roberts – 1 month
Karista Hudelson, MS student – 12 months
Matt Kovach, MS student -12 months
Ben Barst, MS student – 3 months
James Smith, PhD student - 12 months
David Baxter, PhD student – 3 months

11. Collaborations
National Park Service – Field research site at Rocky Mountain National Park
University of Colorado Mountain Research Station – Field research sites
Dr. Andrew Mount, Clemson University – NMR/cellular distribution work
Dr. Nandika D'Souza, University of North Texas – biomaterials group
Dr. Guido Verbeck, University of North Texas – chemistry/mass spec
Dr. Barney Venables, University of North Texas – chromatography
21st Century Biochemicals – protein sequencing

12. Publications
MJ Kovach. 2010. Adaptive significance of carotenoid pigments in alpine copepods. Master's Thesis. University of North Texas. 51pg.

K Hudelson. 2011. Carotenoproteins role in UV tolerance in alpine copepods. Master's Thesis. University of North Texas.

Manuscripts from theses are in preparation

Presentations acknowledging AFOSR funding
(Invited) AP Roberts. Pigments, PAHs, and UV: The "eco" of ecotoxicology. Departmental Seminar. Institute of Environmental Toxicology. Clemson University.

K Hudelson, B Barst, JD Smith, and AP Roberts**.** 2011. Effects of Carotenoprotein expression on UV tolerance in high elevation copepods. Ecological Society of America Annual Meeting. Austin, TX.

K Hudelson, B Barst, JD Smith, and AP Roberts. 2011. Effects of Carotenoprotein expression on UV tolerance in high elevation copepods. South Central SETAC Regional Meeting. Denton, TX.

N Wallace, JD Fox, K Hudelson, AP Roberts, and G Verbeck. 2011. Nanomanipulation-Nanospray Ionization-Mass Spectrometry Coupled to Raman Microscopy to Elucidate Astaxanthin-Protein Interaction in Freshwater Copepods. ACMS Annual Meeting.

(Invited) AP Roberts. 2010. PAHs, Mercury, and Nanoparticles: The Ecotoxicology of Energy. Departmental Seminar. Institute of Arctic and Alpine Research. University of Colorado. (Invited).

MJ Kovach, BD Barst, KE Hudelson, JD Smith, and AP Roberts. 2010. Role of Carotenoids in Ameliorating PAH-Photoinduced Toxicity. National Park Service Research Meeting. Estes Park, CO.

(Invited) AP Roberts, MJ Kovach, B Barst, K Hudelson, J Smith, BJ Venables, and WT Waller. 2010. Plankton and Planes: Pigment Control in Alpine Copepods. National Park Service Research Meeting. Estes Park, CO.

K. Hudelson, Kovach, M. and AP Roberts. 2010. Carotenoproteins reduce phototoxicity in alpine copepods. 2010 SETAC Europe Annual Meeting. Seville, Spain.

(Invited) AP Roberts. 2009. PAHs, Mercury, and Nanoparticles: The Ecotoxicology of Energy. Departmental Seminar. University of Colorado-Denver. Denver, CO.

Kovach, M. and AP Roberts. 2009. Protecting One Bird with Multiple Stones: The Role of Astaxanthin in Alpine Zooplankton Ecology. 2009 SETAC North America Annual Meeting. New Orleans, LA.

Kovach, M. and AP Roberts. 2009. Protecting One Bird with Multiple Stones: The Role of Astaxanthin in Alpine Zooplankton Ecology. 2009 UNT Graduate Research Day, Denton TX.

13. Interactions/Transitions

Over the past two years, I have given several seminars at various universities and meeting presentations on our research. I also presented a talk open to the public on our work at Rocky Mountain National Park. The National Park Service has used our work several times in public outreach presentations, including an interview to National Public Radio. We have been involved with the research community at the University of Colorado Mountain Research Station and mentored an undergraduate student in the CU NSF-Research Experience for Undergraduates program.